RESEARCH ARTICLE

Proteome analysis of combined effects of androgen and estrogen on the mouse mammary gland

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We and other investigators have previously shown that estrogen and androgen have synergistic effects on the growth of mammary epithelial ducts and alveoli in the Noble mouse. However, the molecular mechanisms behind the synergy are unknown. In the present study, we treated female FVB mice with 17-estrodial (E2) and 5-dihydrotestosterone-bezonate (DHT-B) using slow-releasing hormone pellets for 7 months. Dissection showed that hormone treatment caused atypical hyperplasia of mammary ducts and alveoli. A functional proteomic approach was used to study the holistic protein changes in mammary glands. 2-DE was used to separate proteins. Twenty-five protein spots that were differentially expressed in hormone-treated tissues compared to the control were identified by MALDI-TOF-MS, and ESI-quadrupole-TOF-MS, which include some proteins that are correlative with response to estrogen and androgen stimulation, cells differentiation and growth, signal transduction, metabolism, *etc.* Real-time RT-PCR was carried out to verify the different expression. These results offered some clues to understand the function of E2 and DHT-B.

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1 Introduction

Increasing numbers of women are using hormone replacement therapy (HRT) in their 50s and 60s. There is considerable evidence that ovarian hormones, estrogen and possibly progesterone, play an essential role at all stages in the development of human breast cancer in females [1, 2]. HRT significantly increases the risk of developing breast cancer with combinations of estrogen/progestogen increasing the risk to a greater degree than estrogen alone [3, 4], although literature about whether elevated circulating estrogens are related to the development of breast cancer has always been inconsistent [5]. On the other hand, androgens have also been implicated as a possible carcinogenic factor in breast cancer [6]. Administration of several forms of androgens at low doses for short periods of time may prevent or reduce estrogenic cancer risk in the treatment of girls and women with ovarian failure [7, 8]. However, long-term treatment of both male and female rats with moderate to high doses of androgens, including testosterone propionate (TP), 5-dehydrotestosterone (DHT), or dehydroepiandrosterone (DHEA), has been shown to be associated with development of breast cancer [9].

In recent years, researchers have found that administration of estrogen and androgen could simultaneously increase carcinogenesis compared to estrogen or androgen alone [10, 11]. Liao *et al.* [10] found that male Noble (Nb) rats simultaneously treated with 17-estrodial (E2) and testosterone propionate (TP) pellets developed palpable, invasive mammary adenocarcinomas at 100% frequency 5–6 months after the hormonal treatments [10]. Soon afterwards, Xie *et al.* [11] reported similar findings in female Nb rats. However, animals receiving E2 alone for the same time period developed only hyperplasia and, occasionally, microscopic adenomas, while rats receiving TP alone showed only aprocrine altera-

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tions and apoptosis of the epithelial cells [10, 11]. Therefore, TP is likely to function as a potent promoting agent to facilitate estrogen-initiated mammary carcinogenesis in this Nb rat model. Unlike rats, mice are generally considered to be refractory to estrogen-induced mammary carcinogenesis. We treated FVB mice with E2 and esterized DHT-benzoate, alone and in combination, to study the chronic effects of these hormones on the mammary gland [12]. We found that DHT-B alone induced growth and secretion of mammary ductal cells, as well as growth of mammary stroma; E2 alone stimulated much more pronounced growth of both ductal cells and alveolar cells and secretion of alveolar cells, but had no effect on mammary stroma; treatment with both E2 and DHT-B caused more severe hyperplasia of mammary ducts and alveoli, compared with the treatment with each hormone alone. From the results we concluded that E2 and DHT-B have synergistic effects on the growth of mammary epithelial ducts and alveoli. However, the molecular mechanism behind the effect of hormone treatment is unknown.

In general, mRNA-based screens depending on differential expression techniques were used. However, these techniques are not necessarily comprehensive in the context of gene expression at the protein level due to post-transcriptional control and PTMs. An alternative approach is direct screening of the protein profiles, or proteome, of a sample using 2-DE and MS.

In this experiment, we extracted the total proteins from both mammary gland tissues treated with E2 plus DHT-B for 7 months and the control (without hormone treatment), and the differential proteome expression analysis was performed using 2-DE and MS. Four different expressed proteins were analyzed at the mRNA level. Initially, we studied the effect of androgen and estrogen on the mouse mammary gland using proteomics, and identified particular proteins of interest as markers of hyperplasia of mammary epithelial ducts and alveoli, and which offered some clues for understanding the function of E2 and DHT-B.

2 Materials and methods

2.1 Chemicals

IPG DryStrips (3–10 linear), IPG buffer (3–10 linear), cover fluid, agarose and CBB blue dye purchased from Amersham Pharmacia-Biotech (Uppsala, Sweden). DTT, iodoacetamide, trypsin (proteomics sequencing grade), CHCA and TFA were obtained from Sigma (St. Louis, MO, USA). Acrylamide, bis-acrylamide, urea, glycine, Tris, CHAPS, and SDS were from Amresco (Solon, OH, USA). SYBR green sequence detection reagents and Taq polymerase were from PE Biosystems (USA). ACN was from Chinese National Medicine Group Shanghai Chemical Reagent Company (chromatogram grade). Other chemicals were also from Chinese National Medicine Group Shanghai Chemical Reagent Company (analytical grade).

2.2 Animals

Female FVB mice were treated with hormones as described [12]. After 7 months of treatment, the mammary gland tissues from four E2+DHT-B-treated mice and the controls were collected. The left mammary gland tissues of every mouse were fixed with 10% buffered formalin, followed by paraffin embedding; 5-μm paraffin sections were prepared for hematoxylin and eosin (HE) staining and examined under a Zeiss light microscope. The right mammary gland tissues were mixed and used for proteome analysis.

2.3 Protein sample preparation

Mammary gland tissues were kept in liquid nitrogen for 1 day immediately after weighted. Subsequently, the tissues were ground in a mortar and suspended in lysis buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% NP-40, 2% pharmalyte 3–10, 2 mM PMSF, and centrifuged for 20 min at 12 000 rpm at 4°C. The supernatant was -precipitated with acetone and dried. Protein concentrations were determined using the Bradford assay. All samples were stored at −70°C prior to electrophoresis.

2.4 2-DE

2-DE was performed essentially as described in [13]. The first dimension of IPG-DALT 2-DE was run on an IPGphor IEF system (Amersham Pharmacia Biotech). Solubilized mammary gland proteins (1500 μg) were mixed with a rehydration solution containing 8 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer, pH 3–10 L, 18 mM DTT, 20 mM Tris-base and a trace of bromophenol blue, to a total volume of 350 μL, and applied to IPG DryStrips (pH 3–10 L; 180 × 30 × 0.5 mm). After rehydration for 12 h, IEF was performed automatically to a total of 44 kVh at 20°C. Following IEF separation, the gel strips were equilibrated for 2 × 15 min in an equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue; 0.2% DTT was added to the first equilibration buffer, and in the second equilibration buffer DTT was replaced with 3% iodoacetamide. The second-dimensional run was carried out on a SDS-polyacrylamide vertical slab gels with 1-mm-thick, 13.5% separation and 4.8% stacking gels in a Bio-Rad Protein II electrophoresis apparatus. Condense gel and separate gel were run at 12.5 mA/gel and 25 mA/gel constant current, respectively. A molecular weight marker for M_r calibration was added. After 2-DE, the gels were stained with CBB.

2.5 Image acquisition and analysis

The CBB-stained gels were scanned using a Qinghua ultraviolet scanner at an optical resolution of 300 dpi in transmission model. Spot detection, quantification and matching were performed using PDQUEST software Version 6.1 (Bio-

Rad Laboratories, Hercules, CA, USA). A match set consisting of six images, three for normal and three for treated mouse mammary gland was created, and one image from treated mouse mammary gland was selected as the match set standard for spot matching. To correct for variability due to CBB staining and reflect the quantitative variations of protein spot, the individual spot volumes were normalized by dividing their optical density (OD) values by the total OD values of all the spots present in the gel. Then the three parallel gels were translated into a replicate gel, respectively. The significance of expression difference of protein between treated and control mammary gland tissues was estimated by Student's *t*-test, $p<0.05$, and was done using PDQUEST software. All quantitative and qualitative comparisons were also done using PDQUEST software, followed by the confirmation of manual and whole mount gel checking.

2.6 In-gel digestion of proteins

The CBB-stained protein spots were excised from preparative gels using a punch and placed into 500-μL Eppendorf tubes. The proteins were digested in-gel with trypsin as described by Hellmann *et al.* [14]. Briefly, each spot was destained with 50 μL 50% ACN in 25 mM NH_4HCO_3, incubated at 37°C for 0.5 h and repeated once. Then the dried gels were reduced and alkylated. The gel pieces were digested overnight with trypsin (0.02 g/L) in 25 mM NH_4HCO_3 containing 10% ACN at 37°C. The digests were desalted with ZipTip™ (Millipore, Bedford, MA, USA) according to the manufacture's instructions and then subjected to analyze using MALDI-TOF-MS, or ESI-quadrupole (Q)-TOF.

2.7 MS

2.7.1 MALDI-TOF-MS of the Peptides

2.7.1.1 PMF of the peptides

The tryptic peptide mixtures were eluted in 1 μL of a solution containing 5 mg/mL CHCA, 50% ACN and 0.1% TFA. The solution was then loaded onto a 96-well target plate. The samples were air-dried. Molecular weight information of peptides was obtained using an MALDI-TOF-MS Voyager DE-STR from Applied BioSystems (Framingham, MA, USA), equipped with nitrogen laser and operated in reflector/delay extraction mode and auto acquisition control. Only the PMF with signal intensity more than 4500 were retained. All MALDI-MS spectra were internally calibrated using trypsin autolysis fragments ($[M+H^+]$ ions of 842.5099 and 2211.1046). The peptide masses were entered into MS-FIT software. The database searches were performed using the following values: *Mus musculus* species, protein molecular weight range (1000–100 000) and p*I* range (3–10), trypsin digest (one missed cleavage allowed), cysteines modified by carbamidomethylation, mass tolerance ±50 ppm using internally calibration. The criteria for protein identification were set on at least four matching peptides and more than 14% coverage. The first hit protein was considered positive when it scored over 1000, and the difference in score with the second ranked one more than two orders of magnitude. Tryptic autolytic fragments and contamination were removed from the set of data used for database search.

2.7.1.2 PSD modified by 4-sulfophenyl isothiocyanate

The peptides were modified by 4-sulfophenyl isothiocyanate (SPITC) as described by Chen *et al.* [15], and the parent ions that have been modified were used to obtain sequences. The PSD data were manually interpreted. The derived sequence tags were searched using MS-Edman program from the Protein Prospector software package.

2.7.2 ESI-Q-TOF MS of the peptides

Mass spectra were obtained on a Q-TOF mass spectrometer (Micromass, Manchester, UK) fitted with a nanoESI source (Micromass). For on-line lLC/MS analysis, a Waters CapLc solvent delivery system (Waters) was coupled to the MS according to [16]. The MS/MS data were acquired by the software MassLynx (Micromass) and were converted to PKL files, which comprise the mass values of the precursor ions, the intensity and the mass values of the fragment ions, by the ProteinLynx (Micromass). These PKL files were analyzed using MASCOT software (www.matrixscience.com). Search parameters were set as follows: Database: Swiss-Prot or NCBInr; taxonomy: *Mus musculus*; enzyme: trypsin, allowing up to one missed cleavage. Peptide mass tolerance was 1.2 Da and MS/MS mass tolerance was 0.6 Da; fixed modification parameter was carbamidomethylation, and the variable modification parameters were oxidation (Met), and phosphorylation (Ser, Thr, Tyr). We basically selected the candidate peptides with probability-based MOWSE scores (total score) that exceeded its threshold, indicating a significant (or extensive) homology ($p<0.05$), and referred to them as "hits". The criteria were based on the manufacturer's definitions (Matrix Science) [17]. Proteins that were identified with at least two peptides both showing a score higher than 40, were validated without any manual validation. Those with at least two peptides lower than 40 and higher than 20 were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion. For proteins identified by only one peptide, its score had to exceed 40, and its peptide sequence was systematically checked manually. When there were several hits, the first hit was selected.

2.8 Real-time RT-PCR

Four different proteins, expressed in normal and/or treated mouse breast tissue, were analyzed at the mRNA level. Real-time RT-PCR was used to quantitatively measure the levels of mRNAs of RIKEN cDNA 4931406C07, ubiquinol-cyto-

chrome C reductase, mammary-derived growth inhibitor and epidermal fatty acid-binding protein. The primer pairs used traversed introns and test products were sequenced to confirm specificity before use in these assays. PCR products from all samples were analyzed on agarose gels and positives shown to contain a single PCR product of the size predicted from cDNA. The product sizes of the four genes are 146 bp, 130 bp, 107 bp and 142 bp, respectively. The primers used for PCR were as follows: ubiquinol-cytochrome reductase: sense 5'-AGGTGCCCGACTTCTCTGAC-3', antisense 5'-CACATT TTTGGCCGCATAAG-3'; RIKEN cDNA 4931406C07: sense 5'-AGCAGGGGCAGGTCCATTTC-3', antisense 5'-CGCTC CATCTCGAGGGTTTTTA-3'; mammary-derived growth inhibitor: sense 5'-ACGGGCAGGAGACCACACTAACTA-3', antisense 5'-TCACGCCTCCTTCTCATAA-3'; fatty acid-binding protein, epidermal: sense 5'-GGAAGTGGCGCCTGAT GGA-3', antisense 5'-GTTTTGACCGTGATGTTGTTGC-3'. 18S rRNA was used for control. SYBR green sequence detection reagents (PE Biosystems), Taq polymerase, sense and antisense primers were assayed on an iCycler iQ Detection System (Bio-Rad). The thermal cycling parameters were 1 cycle of 94°C for 2.0 min followed by 42 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The accumulation of PCR product was quantified as described in User Bulletin 2 for the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

3 Results and discussion

3.1 Alterations of the mammary gland

HE staining showed that E2+DHT-B-treated mice developed atypical mammary hyperplasia compared with control mammary glands. The nuclei became inhomogeneous, large in size, and more deep stained color than the controls. The proportion of plasma and nucleus was maladjusted and the cells were hyperplasied into sheets. Some mucus was also found in the cells of the treated tissues (Fig. 1).

3.2 2-DE and the analysis of gel images

To isolate proteins efficiently, the 2-DE was performed with a 13.5% separation gel in the second dimension. To effectively identify as many different proteins as possible, we loaded 1500 μg proteins and stained gels with CBB. The resulting images were analyzed using PDQUEST software. A typical 2-DE proteome spot pattern of treated and control tissue is shown in Fig. 2. Under the same experimental conditions, six gels, three for control and three for treated mammary glands were analyzed. An average of 453 and 425 protein spots were detected in the gels of the treated and control mammary glands, respectively.

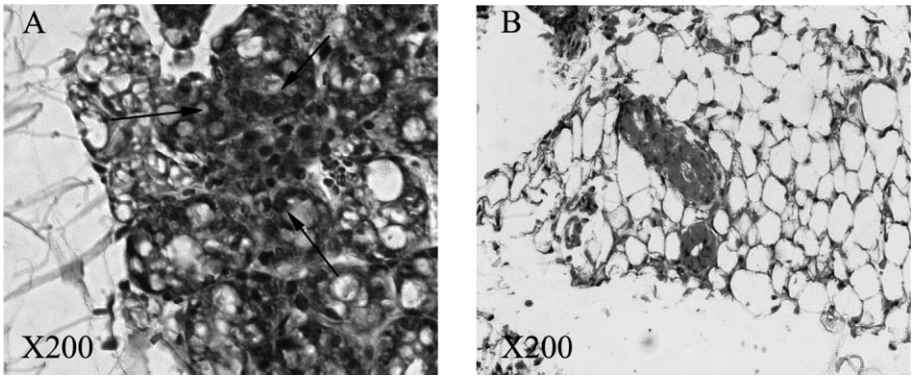

Figure 1. HE staining of mammary glands: (A) mammary tissue from mice receiving E2+DHT-B for 7 months; (B) controls. Results show that the nuclei from treated tissue become inhomogeneous, larger in size, and more deep stained in color than the controls, that the proportion of plasma and nucleus are maladjusted and that the cells hyperplase into a sheet (arrows).

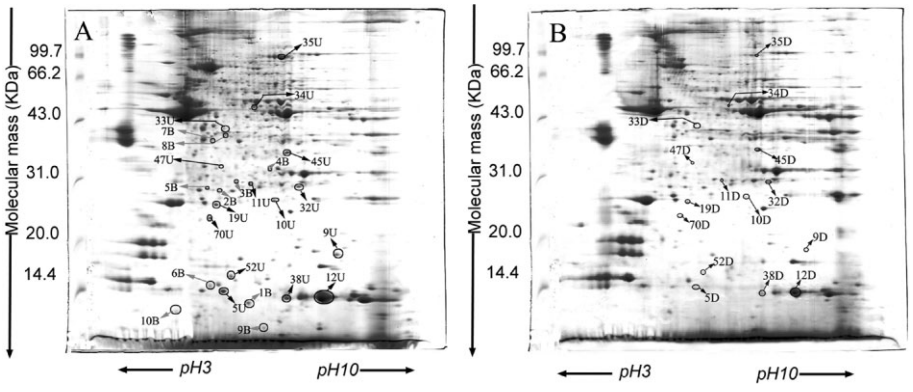

Figure 2. CBB-stained 2-D acrylamide gels of tissue samples (A) treated with E2+DHT-B for 7 months; (B) untreated control. Each sample (1.5 mg) was subjected to 2-DE and CBB staining. Molecular weight of markers are shown on the left. Blank arrows indicate the proteins that are upregulated in treated mammary glands, $p<0.05$; red arrows indicate those that are detected only in the treated samples.

Using gel matching and analysis, the proteomic profile of treated mammary glands and controls were shown to be very similar, as shown in Fig. 2, and the average correlation coefficient between those two patterns was 0.8 by correlation analysis of gels. In the 2-DE maps of the treated and control mammary glands, 51 protein spots were found to be differentially expressed with changes in the stain density of threefold or more, including some up- and down-regulated proteins and completely different proteins ($p<0.05$). Two representative different spots were magnified and are shown in Fig. 3. A representative picture of PDQUEST software analysis was showed in Fig. 4.

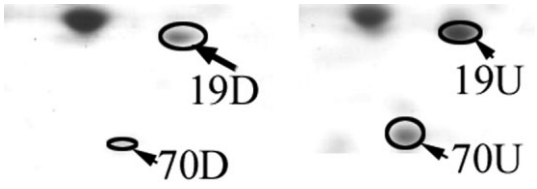

Figure 3. Magnified regions of 2-D images showing spot 19 and 70, which were up-regulated in treated tissues. "D": down-regulated, "U": up-regulated. Left, untreated controls; right, treated tissues.

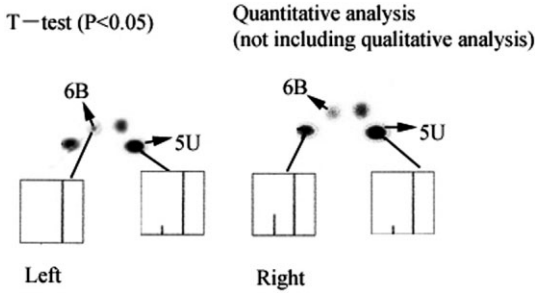

Figure 4. The vertical lines of each graph represent the quantitation in the *t*-test (left) and quantitative analysis (not including the qualitative analysis) in the control replicate gel (left line in each graph) and treated replicate gel (right line in each graph).

3.3 MS analysis and protein identification

Twenty-five differentially expressed spots (including the spots with threefold difference and several spots with less difference) were identified by MALDI-TOF or ESI-Q-TOF MS analysis. Table 1 lists 10 proteins that were detected only in the 2-DE map of treated mammary gland tissues. Table 2 lists 15 proteins that were up-regulated in treated mammary glands compared to the controls ($p<0.05$). All identified different proteins are shown in Fig. 2. The proteins detected only in treated tissues are labeled with red arrows. The other up-regulated spots are marked with black arrows. A representative PMF map and the PSD mass spectrum of mammary-derived growth inhibitor (spot 5U) are shown in Fig. 5A and B. A representative map of peptide sequences analysis of ubiquinol-cytochrome c reductase (spot 32U) analyzed by ESI-Q-TOF is shown in Fig. 6.

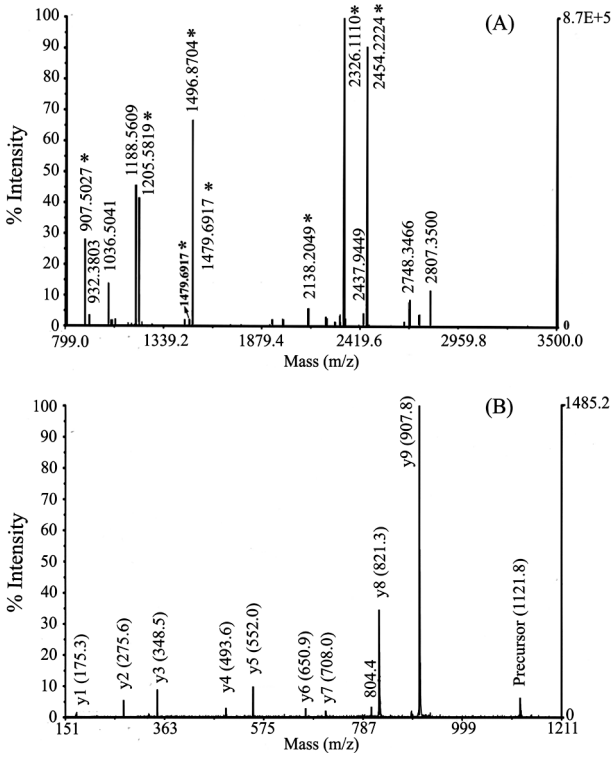

Figure 5. The PMF (A) and PSD (B) of spot 5U obtained by MALDI-TOF.

3.4 Real-time RT-PCR

As described in the introduction, one of the factors for using a protein-based approach in this study was that previous reports have shown that mRNA and protein levels may not correlate well [18, 19]. Therefore, we used real time RT-PCR to compare the relative abundance in mRNA levels for proteins, shown to be up-regulated or only expressed in treated tissue, between treated and control tissue. In the case of fatty acid-binding protein, epidermal (spot 52U), there was a significant increase in mRNA abundance between treated and control samples that correlates with the increase at the protein level. Consistently, the mRNA of RIKEN cDNA 4931406C07 (spot 8B) was only detected in the treated tissues in agreement with the result of protein expression. However, in some instances, the level of mRNA and the expression level of the related protein were not consistent. For example, the mRNA for ubiquinol-cytochrome reductase increased up to 40-fold, much higher than the1.5-fold in the protein expression level between the treated and control samples (Fig. 7, Table 3). Through the comparison of the levels of mRNAs and proteins of the four genes, consistent up-regulations were obtained, although the degrees of the changes were not so consistent in some instances. This could possibly be due to an additional regulation of PTM. The consistent results indicated that E2 and DHT-B influenced the expression of the four genes at the level of transcription.

Table 1. Absolutely different proteins (only expressed in treated mammary gland tissues) ($p<0.05$)[a)]

Spot	Protein description	Accession no.	MW	p*I*	Peptides matched	Coverage
1B	Cytokeratin KRT2–6HF	AAK17206	42 471	5.11	SLDLDSIIAEVK (ion score 43) (+2)	3%
2B	Thioredoxin-dependent peroxide reductase, mitochondrial precursor (perioredoxin 3)	P20108*	28 337	7.15	DYGVLLESAGIALR (ion score 79) (+2) GLFIIDPNGVVK (ion score 37) (+2)	10%
3B	Antioxidant enzyme AOE372 (peroxiredoxin 4)	U96746	31 052.7	6.66		43%
3B	Peroxiredoxin 4	O08807*	31 261	6.67	SINTEVVACSVDSQFTHLAWINTPR (ion score 69) (+3) IPLLSDLNHQISK (ion score 53) (+3) GLFIIDDKGVLR (ions score 55) (+2) QITLNDLPVGR (ions score 54) (+2)	22%
4B	Carbonic anhydrase II	AAH55291	29 138	6.52	SIVNNGHSFNVEFDDSQDNAVLK (ion score 68) (+3) AVQQPDGLAVLGIFLK (ion score 59) (+3)	15%
5B	Cytokeratin KRT2–6HF	AAK17206	42 471	5.11	SLDLDSIIAEVK (ion score 61) (+2)	3%
6B	Cytokeratin KRT2–6HF	AAK17206	42 471	5.11	SLDLDSIIAEVK (ion score 49) (+2)	3%
7B	F-actin capping protein alpha-2 subunit	P47754[a]	32 987	5.58	LLLNNDNLLR (ion score 47) (+2) FTVTPSTTQVVGILK (ion score 52) (+2)	8%
8B	RIKEN cDNA 4931406C07	NP_598493	35 430	5.86	IAEVGGVPYLLPLVNK (ion score 47) (+2) APLVCLPVFVSK (ion score 53) (+2)	8%
9B	Kallikrein	M18614	6 277.9	5.33		49%
10B	TCR alpha-chain V-J-C precursor	S81113	29 530.7	5.90		47%

a) The spots representing the identified proteins are indicated in Fig. 2. Protein database accession numbers were obtained on NCBI database or Swiss-Prot (* is indicated). Sequence informations generated by MS/MS (sequence, ion score, and charge) are indicated. Proteins identified by both MALDI and ESI-Q-TOF were marked with italic numbers.

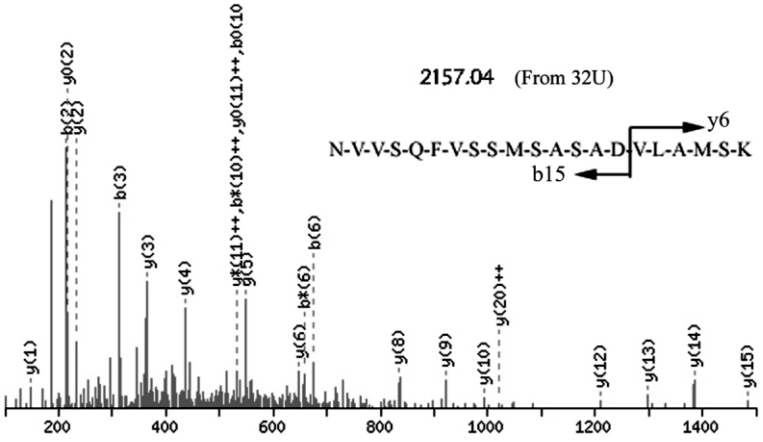

Figure 6. The MS/MS of 2+ ion peptides of spot 32U obtained by ESI-Q-TOF.

3.5 Analysis of the identified differential proteins

The identified proteins, listed in Tables 1 and 2, were uniquely or differentially expressed between the treated and control samples. This was demonstrated using the PDQUEST software, with which the individual spot volumes were normalized by dividing their optical density (OD) values by the total OD values of all the spots present in the gel, thus removing the artificial factors. The three parallel gels from the normal or treated samples were divided into a group, respectively,

Table 2. Up-regulated proteins in the treated mammary gland tissues compared to the controls ($p<0.05$)[a)]

Spot	Protein description	Accession no.	MW	p*I*	Protein alteration[b), c)]	Peptides matched	Coverage
5U	Mammary-derived growth inhibitor	U02884	14 818.9	6.11	↑ (12.3)	1122 (K)SLGVGFATR(Q)	57%
5U	Fatty acid-binding protein, heart (H-FABP) (mammary-derived growth inhibitor)	P11404 *	14 666	6.15	↑ (12.3)	NFDDYMK (ion score 23) (+2) SLGVGFATR (ion score 45) (+2) QVASMTKPTTIIEK (ion score 12) (+3) QVASMTKPTTIIEK (ion score 17) (+2) QVASMTKPTTIIEK (ion score 18) (+3) SLVTLDGGK (ion score 51) (+2) WNGQETTLTR (ion score 60) (+2) ELVDGKLILTLTHGSVVSTR (ion score 12) (+3) LILTLTHGSVVSTR (ion score 73) (+2) LILTLTHGSVVSTR (ion score 54) (+3) LILTLTHGSVVSTR (ion score 61) (+3) LILTLTHGSVVSTR (ion score 91) (+2) LILTLTHGSVVSTR (ion score 64) (+3) LILTLTHGSVVSTR (ion score 60) (+2)	52%
9U	Unknown	X52803	17 971.4	7.73	↑ (8.3)		42%
10U	Proteasome subunit beta type 2	Q9R1P3*	23 063	6.52	↑ (3.5)	NGYELSPTAAANFTR (ion score 23) (+2) TPYHVNLLLAGYDEHEGPALYYMDYLAALAK (ion score 44) (+4) APFAAHGYGAFLTLSILDR (ion score 54) (+3) APFAAHGYGAFLTLSILDR (ion score 16) (+3) FILNLPTFSVR (ion score 40) (+2) VIDKDGIHNLENIAFPK (ion score 17) (+3)	46%
19U	Ferritin light chain	J04716	20 802.5	5.66	↑ (4.0)		51%
19U	Ferritin light chain 1	P29391*	20 671	5.66	↑	QNYSTEVEAAVNR (ion score 61) (+2) DDVALEGVGHFFR (ion score 76) (+2) LLEFQNDR (ion score 42) (+2) NLNQALLDLHALGSAR (ion score 60) (+3) NLNQALLDLHALGSAR (ion score 52) (+3) NLNQALLDLHALGSAR (ion score 81) (+2) NLNQALLDLHALGSAR (ion score 49) (+3) ADPHLCDFLESHYLDK (ion score 13) (+3) VAGPQPAQTGAPQGSLGEYLFER (ion score 81) (+3) VAGPQPAQTGAPQGSLGEYLFER (ion score 24) (+3)	48%
34U	Cytosolic acyl coenzyme A thioester hydrolase, inducible	O55137*	46 335	6.12	↑ (10.0)	SCWDEPLSIAVR (ion score 28) (+2) DVQTPFVVELEVLDGHEPDGGQR (ion score 78) (+3) GFAVMALAYYNYDDLPK (ion score 14) (+2) GPGIGLLGISK (ion score 31) (+2) GGELGLAMASFLK (ion score 67) (+2) SDTTFLFLVGQDDHNWK (ion score 1) (+3)	22%
35U	62-kDa subunit of TFIIH	AJ002366	61 851.7	8.88	↑ (10.0)		14%
38U	Beta-globin major	J00413	15 840.3	7.13	↑ (5.0)		65%
47U	PA28 alpha subunit	D87909	28 673.1	5.73	↑ (4.6)		42%
52U	Fatty acid-binding protein, epidermal (E-FABP) (keratinocyte lipid-binding protein)	Q05816*	15 470	6.14	↑ (8.1)	ELGVGLALR (ion score 32) (+2) TTVFSCNLGEK (ion score 18) (+2) TTVFSCNLGEKFDETTADGR (ion score 57) (+3) MIVECVMNNATCTR (ion score 74) (+2)	31%
52U	Keratinocyte lipid-binding protein	X70100	15 137.5	6.14	↑		53%

Table 2. Continued

Spot	Protein description	Accession no.	MW	p*I*	Protein alteration[b), c)]	Peptides matched	Coverage
70U	Ferritin heavy chain	P09528*	21 093	5.53	↑ (5.8)	IFLQDIK (ion score 18) (+2) LATDKNDPHLCDFIETYYLSEQVK (ion score 23) (+3) LATDKNDPHLCDFIETYYLSEQVK (ion score 19) (+4) LATDKNDPHLCDFIETYYLSEQVK (ion score 6) (+4)	17%
11U	Small heat shock protein	L07577	23 013.9	6.12	↑ (2.3)		32%
12U	Beta-globin major	J00413	15 840.3	7.13	↑ (1.9)		59%
32U	Ubiquinol-cytochrome c reductase	NP_079986	29 634	8.91	↑ (1.5)	VPDFSDYR (ion score 36) (+2) NVVSQFVSSMSASADVLAMSK (ion score 85) (+2) EIDQEAAVEVSQLR (ion score 70) (+2) EIDQEAAVEVSQLRDPQHDLDR (ion score 10) (+3) EIDQEAAVEVSQLRDPQHDLDR (ion score 28) (+3) KGPAPLNLEVPAYEFTSDDVVVVG (ion score 19) (+2)	27%
33U	Transaldolase	Q93092*	37 534	6.57	↑ (1.6)	LFVLFGAEILK (ion score 32) (+2) ALAGCDFLTISPK (ion score 73) (+2)	7%
45U	Insulin-like growth factor binding protein 5	U02026	30 243.1	8.58	↑ (2.4)		38%

a) The spots representing the identified proteins are indicated in Fig. 2. Protein database accession numbers were obtained on NCBI database or Swiss-Prot (* is indicated). Sequence information generated by MS/MS (sequence, ion score, and charge) or MALDI (underlined) are indicated. Proteins identified by both MALDI and ESI-Q-TOF were marked with italic numbers.

b) ↑, up-regulated in treated mammary glands.

c) Protein mean abundance (ppm) treated mammary/control, which were calculated from replicate gel from three gels for each sample.

Table 3. Comparison of the results from proteomics and genomics

Protein or gene name	Regulated in treated tissue through proteomics	Regulated in treated tissue through genomics
Ubiquinol-cytochrome C reductase	Up-regulated 1.5-fold	Up-regulated 42.8-fold
RIKEN cDNA 4931406C07	Only	Only
Mammary-derived growth inhibitor	Up-regulated 12.3-fold	Only
Fatty acid-binding protein, epidermal	Up-regulated 8.1-fold	Up-regulated 19.7-fold

and the matched spots in the three parallel gels were averaged, respectively. Only the spots with statistically significant difference were accepted. Every different spot was confirmed by manually checking. In total, 25 protein spots that met these criteria were identified. They include either uniquely expressed (such as perioredoxin 3; kallikrein, etc.) or up-regulated (such as mammary-derived growth inhibitor, ferritin light chain) proteins in the treated mammary gland.

According to their function, these differentially expressed proteins can be classified into eight categories (Table 4). Three of the identified proteins were related to hormone stimulation directly. Spot 9B was identified as kallikrein. Luo *et al.* [20] found that the kallikrein 10 gene expression was up-regulated by estrogen, androgen and progestin stimulation through their own receptors in the breast cancer cell lines BT-474, MCF-7 and T-47D. Yousef *et al.* [21] found that kallikrein 5 is a potential serum biomarker for breast and ovarian cancer because it is almost undetectable in serum of normal individuals (male and female) and patients with other diverse malignancies. However, higher concentration of this protein was detected in a proportion of patients with ovarian (69%) and breast (49%) cancer. Spot 45U was identified as insulin-like growth factors binding protein (IGFBP) 5.

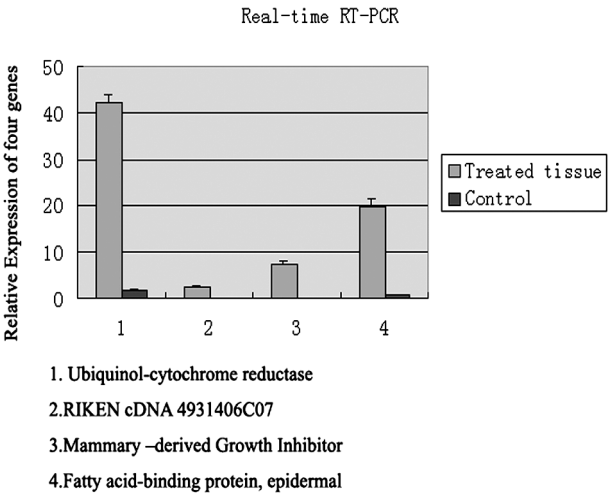

Figure 7. Real time RT-PCR quantification of mRNA levels of ubiquinol-cytochrome reductase, RIKEN cDNA 4931406C07, mammary-derived growth inhibitor, epidermal fatty acid-binding protein in treated and controls. The experiment was repeated for three times.

IGFBPs (designated as IGFBP-1 to IGFBP-6) are a family of proteins that specifically bind IGF-I and IGF-II receptors [21]. Yu *et al.* [22] found that estrogens or androgens increased the effect of IGF-I on breast cancer cells by stimulating the expression of IGF-I, and IGF-I receptor, and a synergistic effect on breast cancer risk was suggested for IGF-I or IGFBP-3 with estrone or testosterone among both pre- and postmenopausal women. Spot 11U (up-regulated in treated mammary gland) was identified as small heat shock protein (HSP25) similar to HSP27, which is up-regulated in breast cancer cells, estrogen receptor-positive cells and breast tumor biopsy specimens [23]. HSP25 can be induced by heat-shock and estrogen treatment through its putative transcription factor-binding elements (including two heat-shock elements, and an estrogen-responsive element half-site) in its promoter region [24]. This set of proteins suggests that E2 and DHT-B maybe affect mammary glands initially through its receptors, and receptor-related proteins.

Three proteins were identified to be related with the redox system, including peroxiredoxin 4 (spot3B), thioredoxin-dependent peroxide reductase, mitochondrial (precursor) (spot2B), and ubiquinol-cytochrome c reductase (spot 32U). Peroxiredoxin 4 is probably involved in redox regulation of the cell. The peroxiredoxin family not only serves as a peroxidase, but also functions in detoxification of oxidants, cell proliferation, differentiation and gene expression [25]. Crowley-Weber *et al.* [26] found that peroxiredoxin 4, TRAF2, thioredoxin peroxidase (peroxiredoxin) 2, which may play a role in apoptosis and early stage carcinogenesis, were identified as up-regulated proteins in cancer cell lines. Thioredoxin-dependent peroxide reductase, which involves in redox regulation of the cell and protects radical-sensitive enzymes from oxidative damage by a radical-generating system, was identified only in oncogenic mammary glands. This protein seems to be sensitive to oxygen, and forms a thioredoxin-dependent system to eliminate reactive oxygen species [27]. It is possible that the cells of treated tissue up-regulated these proteins to compensate the accelerated growth.

Table 4. The function classify of the identified proteins significantly different between the treated and controls

Spot number	Protein description
Proteins related with hormone	
9B	Kallikrein
45U	Insulin-like growth factors binding protein (IGFBP) 5
11U	Small heat shock protein
Proteins related with redox	
2B	Thioredoxin-dependent peroxide reductase
3B	Peroxiredoxin 4
32U	Ubiquinol-cytochrome c reductase
Proteins related with metabolism	
4B	Carbonic anhydrase II
19U	Ferritin light chain
33U	Transaldolase
70U	Ferritin heavy chain
Proteins related with fatty acid	
5U	Mammary-derived growth inhibitor
52U	Fatty acid-binding protein, epidermal (E-FABP)
34U	Cytosolic acyl coenzyme A thioester hydrolase, inducible
Proteins related with proteasome	
10U	Proteasome subunit beta type 2
47U	PA28 alpha subunit
Proteins related with immune response	
10B	TCR alpha-chain V-J-C precursor
38U, 12U	Beta-globin major
Proteins related with cytoskeleton	
7B	F-actin capping protein alpha-2 subunit
35U	62-kDa subunit of TFIIH
Unknown function proteins	
1B, 5B, 6B	Cytokeratin KRT2–6HF
8B	RIKEN cDNA 4931406C07
9U	Unknown

Four proteins related to the metabolism were also identified, ferritin light chain (spot 19U), ferritin heavy chain (spot 70U), carbonic anhydrase II (spot 4B), and transaldolase (spot33U). Ferritin light and heavy chains were up-regulated in the treated mammary gland. Ferritin is the major iron-storage protein, which sequesters and detoxifies excess iron that is taken up by cells but is not utilized in normal metabolic processes. Human ferritin consists of various combinations of heavy (FerH, M_r 21 000) and light (FerL, M_r 19 000) chains, and excess iron leads to an increase in the synthesis of both heavy and light chains. It is well known that iron plays an essential role in many biochemical reactions, and that rapidly growing cells require more iron for their growth and metabolism than resting cells. They may be useful for assessing prognosis and guiding therapeutic decisions in breast cancer patients [28, 29]. Carbonic anhydrase II catalyses the reversible hydration of carbon dioxide. Since tumor cells require a high bicarbonate flux for their growth, it would be reasonable for the carbonic anhydrase II to be up-regulated.

Mammary-derived growth inhibitor (spot 5U) and epidermal fatty acid-binding protein, (Spot 52U) belong to the fatty-acid binding protein family [30]. In mammary gland, these proteins are involved in cell signaling, growth inhibition and differentiation. Krieg *et al.* [31] found that epidermal fatty acid-binding protein was up-regulated in both the benign papilloma and the malignant mouse skin squamous cell carcinomas stages during tumor development.

Proteasome subunit beta type 2 (10U) and PA28 alpha subunit (47U) are two related proteins. Proteasomes play central roles in transcriptional regulation, cell cycle and apoptosis. It is interesting to speculate that up-regulation of particular subunits of the proteasome complex may be a mechanism by which hormone causes mammary hyperplasia. Indeed, in breast tumors, proteasome subunit, beta type 3 (PSMB3) is coexpressed with the oncogene tyrosine kinase-type cell surface receptor, ERBB2 which is amplified [32]. PA28 is composed of three homologous subunits, alpha and beta and gamma. PA28-gamma was found to be over-expressed in thyroid cancer, especially in its growth-accelerated cells [33].

The other identified proteins include proteins related with immune response, such as beta-globin major and TCR alpha-chain V-J-C precursor. The cells may up-regulate these immune proteins to protect against the affect of hormone.

In addition to the known functional proteins mentioned above, we also identified some proteins of unknown function in public database. These include RIKEN cDNA 4931406C07 (spot 8B), cytokeratin KRT2–6HF (spot 1B, 5B and 6B), 62-kDa subunit of TFIIH, and a no name given protein (spot 9U).

From the result of the dissection, only atypical hyperplasia of mammary was observed, and no mammary tumors were observed after 7 months of treatment with both E2 and DHT-B. However, we found several proteins that were only expressed or up-regulated in tumors reported by others (as discussed above). It is possible that proteome analysis is more sensitive than the method of histopathology for the observation of carcinogenesis. It may be possible to find mammary tumors if the mice are treated for a longer time with these hormones.

Only about 450 proteins were detected in the gels in this study, which is much lower than the number for the whole mammary gland proteins. Only 25 differentially expressed proteins with high abundance were identified. It is highly possible that many important differentially expressed proteins with low abundance were not identified in this study. One main reason is that the 2-DE gels were stained with CBB, and many proteins with low abundance would not be detected. For a more comprehensive analysis, more proteins could be visualized by separation on a series of narrower pH range IPG strips and by silver or fluorescence stain, thereby increasing loading capacity and sensitivity. Future studies will also require preselection of proteins by fractionation of cellular organelles. As an alternative to 2-DE, such enriched fractions could be analyzed by LC-MS/MS that has the capability of detecting low abundance proteins in complex mixtures [34].

In summary, this is the first study using functional proteomics to profile mammary glands before and after treatment with E2 and DHT-B. E2 and DHT-B treatment led to changes of genome and proteome, and caused atypical hyperplasia of mammary ducts and alveoli. We have shown that the expressions of various proteins were altered with hormone treatment. These results suggest that E2 and DHT-B could affect mammary glands through hormone receptors, and the related proteins. Our study offers some clues for understanding the function of E2 and DHT-B, which should be helpful in understanding the effect of these hormones on mammary glands, and could lead to the discovery of proteins that play functional roles in the carcinogenesis, and, particularly, the discovery of markers of tumor progression.

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